Social Stress Causes Extramedullary Hematopoiesis in the Spleen

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Abstract

Chronic psychological stress promotes hematopoietic production of proinflammatory innate myeloid cells, such as monocytes and neutrophils. Enhanced production of proinflammatory myeloid cells is tightly linked to negative health outcomes in both high stress populations. Circumstances with leukocyte demand (e.g., infection, cancer, blood loss) hematopoiesis may occur outside of the bone marrow, such as within the spleen. Mechanisms of enhanced myelopoiesis with stress are explored here in a mouse model of psychosocial stress. In vitro colony forming unit (CFU) assays were performed on bone marrow, blood, and spleen following six cycles of repeated social defeat (RSD) stress. RSD increased colony forming units in the blood and spleen but not the bone marrow. However, the phenotype of CFU in the bone marrow was shifted from erythropoietic to myelopoietic progenitors. Taken together these results show that RSD skewed bone marrow hematopoiesis towards myelopoiesis, and that RSD increased extramedullary hematopoiesis in the spleen. For the first time, this study demonstrates that social stress promotes extramedullary hematopoiesis as a novel mechanism of stress-induced myelopoiesis.

Introduction

Broadly, chronic psychosocial stress is associated with a sequela of immunological changes that are often correlated with poor health outcomes. Many of these immunological changes are related to increased accumulation of primed monocytes that have increased potential for inflammatory signaling [1, 2] and are resistant to the anti-inflammatory effects of glucocorticoids (GCs) [3, 4]. Moreover, many of the pro-inflammatory effects of stress can be attributed to enhanced monocytopoiesis in the bone marrow that results in the selective accumulation of the Ly6C^{hi} monocyte subset [2, 5]. Ly6C^{hi} monocytes have a higher inflammatory capacity compared to their more mature immunoregulatory Ly6C^{lo} counterparts [6, 7]. Additionally, there is evidence that this monocytic immune activation contributes to psychiatric illness in humans, as reviewed by Beumer et al. [8]. For example, increased perivascular brain-macrophages were observed in depressed patients who committed suicide [9]. Moreover, PTSD symptoms significantly correlated with pro-inflammatory NFκB signaling in leukocytes and with GC-resistance in monocytes [10, 11]. Thus, these clinical data provide key evidence that links stress, monocytes, health, and mood.

Repeated social defeat (RSD) in mice recapitulates key immunological and behavioral deficits [12, 13] associated with psychosocial stress in humans. For example, RSD increased monocytopoiesis in the bone marrow that caused selective accumulation of Ly6C^{hi} monocytes in circulation, spleen, and brain [14, 15]. The accumulation of Ly6C^{hi} monocytes during RSD promoted a pro-inflammatory leukocyte "transcriptional fingerprint" that was similar to that observed in human populations [2]. Similarly, RSD promotes a primed monocyte phenotype characterized by exaggerated inflammatory response to *ex vivo* innate immune challenge that is resistant to inhibition by GCs [16]. Additionally, the development of prolonged anxiety-like

behavior that is detectable up to 8 days after RSD [17] is dependent upon sympathetic activation of the immune system [2, 14, 16]. Further studies revealed that the development of prolonged anxiety-like behavior was specifically dependent on monocyte accumulation in the brain following RSD [18]. Taken together, monocyte trafficking to the brain represent a novel axis of immune-to-brain signaling that promotes prolonged behavioral responses to stress [19, 20].

Other circumstances involving infection, cancer, or blood loss are associated with increased production of proinflammatory myeloid cells that is enhanced by extramedullary myelopoiesis within the spleen [21]. The potential for extramedullary hematopoiesis following stress was assessed in this study.

Materials and Methods

Mice: Male C57BL/6 (6-8 weeks old) and male CD-1 retired breeder mice were purchased from Charles River Breeding Laboratories (Wilmington, MA), and allowed to acclimate to their surroundings for 7-10 days before experiments. Mice were housed in 11.5"x 7.5"x 6" polypropylene cages. Rooms were maintained at 21°C under a 12 h light: 12 h dark cycle from 0600-1800 h with *ad libitum* access to water and rodent chow. All experiments took place between 0800 and 1100 h, unless otherwise noted. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and were approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

Repeated Social Defeat (RSD). RSD was performed as previously described [18]. In brief, an aggressive male intruder CD-1 mouse was introduced into cages of established male cohorts (3 per cage) of C57BL/6 mice for 2 hours between 17:00 and 19:00 for six consecutive nights. During each cycle, submissive behavior including upright posture, fleeing, and crouching were observed to ensure defeat of the resident mice. If the intruder did not initiate an attack within 5-10 minutes or was attacked by any of the resident mice, a new intruder was introduced. At the end of the 2 h period, the intruder was removed, and the residents were left undisturbed until the following day when the protocol was repeated. Different intruders were used on consecutive nights. The health status of the mice was carefully examined throughout the experiment. Mice that were injured or moribund were removed from the study. Consistent with previous studies using RSD [15], less than 5% of mice met the early removal criteria. Control mice (CON) were left undisturbed in their home cages.

Colony forming unit assay. To determine the presence and number of myeloid colony-forming units (CFU) in whole tissue, cells will be cultured in appropriate complete methylcellulose-based medium (e.g., MethoCult GF M3434 from STEMCELL Technologies) in six-well plates. Colony numbers and morphology will be determined after 10-14 days in culture.

Statistical analysis. To determine significant main effects and interactions between main factors, data were analyzed using two-way ANOVA using the General Linear Model procedures of SAS (Cary, NC). ANOVA results are presented in figure legends. When there was a main effect of experimental treatment or a treatment interaction effect, differences between means were evaluated by an *F*-protected t-test using the Least-Significant Difference procedure of SAS. All data are expressed as treatment means ± SEM.

Fig 1. Increased colony forming cells in blood and spleen 14 hours after RSD

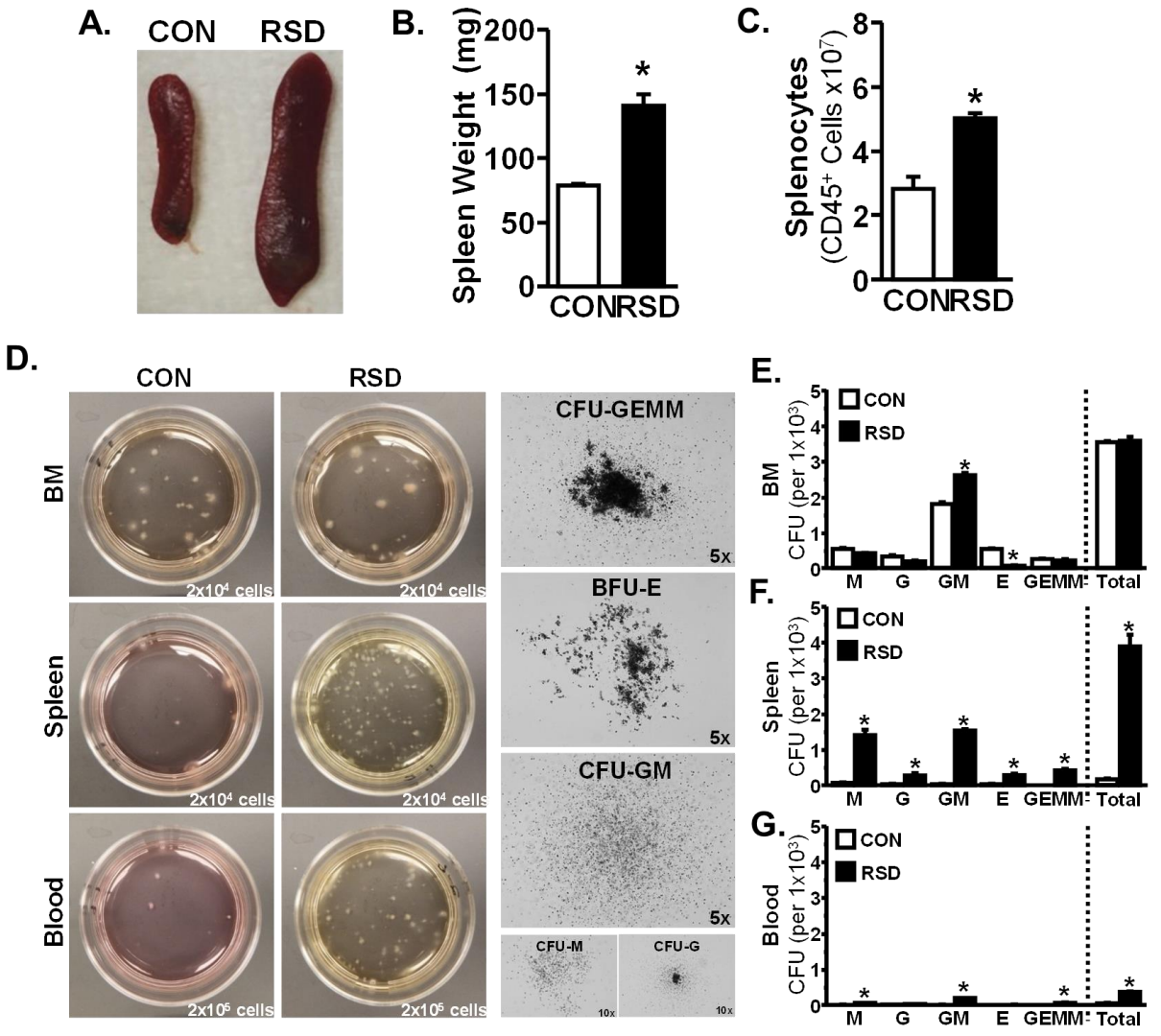

Figure 1. RSD increased CFU in spleen and blood. Male C57BL/6 mice were exposed to 6 cycles of RSD and 14 hours later bone marrow (BM), spleen, and blood cells were plated in MethoCULT media. **A)** Representative images taken after 10 days in culture. **B)** Representative images of hematopoietic colonies. **C-E)** Enumeration of colonies. Bars represent the mean ± SEM. Means with asterisk (*) are significantly different from CON ($p<0.05$)

Results

RSD increased CFU in blood and spleen and is associated with shifted myelopoiesis in bone marrow

Fourteen hours following RSD, hematopoiesis was assessed in blood, spleen, and bone marrow. RSD increased spleen size, and this was evident by simple visual inspection (Fig. 1A) and was associated with significantly increased spleen weight (Fig. 1B) and increased number of splenocytes (Fig. 1C). Next, CFU were determined. CFU can be categorized into different phenotypes by microscopy (Fig. 1D). From visual observation it was apparent that RSD dramatically increased CFU in blood and spleen but not BM (Fig. 1D). Indeed, the total number of BM CFU was unaltered by RSD but the phenotype was shifted from CFU E to CFU GM (Fig. 1E). Fig. 1F shows that RSD significantly increased all phenotypes of CFU, and Fig. 1G shows that RSD significantly increased CFU M, GM, and GEMM in blood.

Next, to assess in situ proliferation of progenitors within the spleen following RSD, BrdU was injected 30 minutes prior to tissue collection, and tissue was sectioned and labeled for BrdU. Consistent with increased extramedullary hematopoiesis in the spleen, there was a substantial increase in the number of BrdU+ cells in the RSD spleens (Fig. 2). Notably, this increase was localized specifically to the red pulp and not the white pulp.

Fig 2. RSD causes proliferation within the splenic red pulp

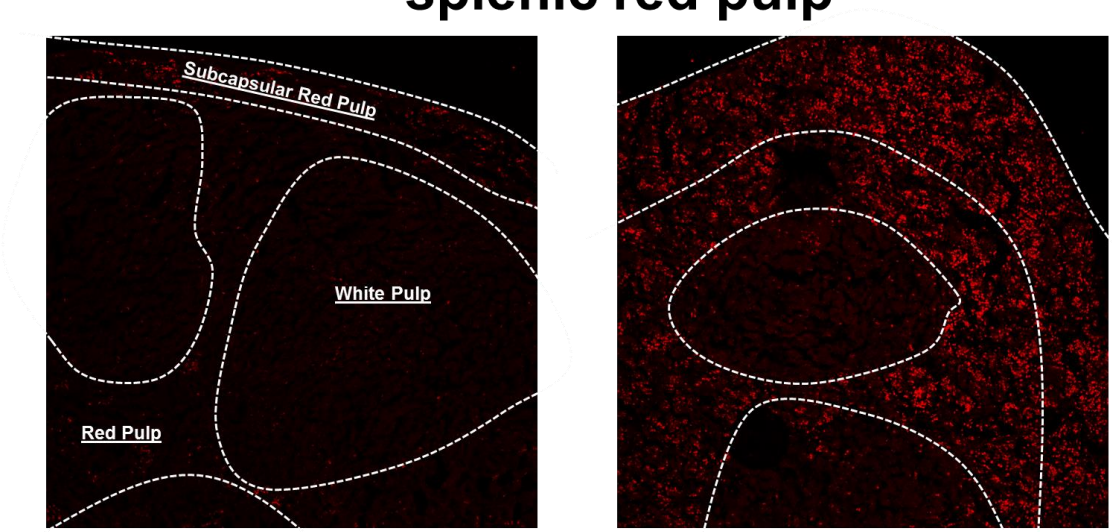

Figure 2. RSD causes proliferation within the splenic red pulp. Male C57BL/6 mice were exposed to 6 cycles of RSD and 14 hours later were injected with BrdU (50 mg/kg). Mice were sacrificed 30 minutes after BrdU injection and were perfusion fixed. BrdU labeling in the spleen is displayed. Spleen subanatomy is depicted on the image (red pulp, white pulp, and subcapsular red pulp).

Discussion

The results presented here demonstrate a novel and critical role for the spleen in the maintenance of increased myelo9poiesis following social stress. First, it was observed that RSD significantly increased spleen size, weight, and splenocytes. This splenomegaly was associated with a substantial increase in the number of CFU in the spleen. This increase was particularly evident in the CFU GM, M, and GEMM subtypes. Moreover, increased CFU in blood were detected. This likely indicates that there was increased release of progenitors from the bone marrow that transited through circulation, deposited in the spleen, and expanded there. Notably, no change in the total number of CFU in the BM was detected. However, increased CFU GM

and decreased CFU E were detected. This likely represents a skewing of hematopoiesis away from erythropoiesis and towards myelopoiesis that resembles our previous findings [2]. In line with these collective observations, BrdU pulse chase labeling revealed that RSD substantially increased the number of proliferating cells within the spleen. This increase was specifically localized to the red pulp and not the white pulp. This is in line with previous observations regarding the sub anatomical localization of extramedullary hematopoiesis in the spleen [21]. Overall, these data demonstrate that RSD causes a substantial extramedullary hematopoiesis response in the spleen.

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